

## USE OF TRIPLEX STRUCTURE DNA IN TRANSFERRING NUCLEOTIDE SEQUENCES

The present application relates to the use of DNA sequences which are capable of having a triple-stranded structure or organisation (known as triplex DNA) for transferring nucleotide sequences into cells, and to recombinant vectors containing such triplex sequences.

5        Thus the invention concerns the definition and provision of novel means which can be used, for example, in the context of protocols for gene therapy or transgenesis for the production of transgenic animals or plants or recombinant cells or cell lines. Such means comprise producing novel vectors which can transfer a nucleotide sequence, in particular a sequence of therapeutic interest, into target cells in the human or animal body.

10        An important limitation to current gene therapy approaches lies in the vectorisation of the gene of therapeutic interest. Retroviral vectors derived from an oncovirus, principally from MoMLV, have been widely used for gene transfer. Their application is largely limited by the fact that oncoviruses only integrate into target cells which are actively dividing. In contrast, lentivirus have the unique capacity among retroviruses of infecting differentiated non mitotic cells and  
15        represent viral candidates of interest for the development of novel vectors. While retaining the advantages of an oncoviral vector (absence of immunogenicity, stable integration), lentiviruses could enable in vivo transduction of non mitotic differentiated tissues (brain, muscle, liver, lung...) and could therefore have a wide range of applications in gene therapy.

20        Different attempts at constructing retroviral vectors from lentiviruses have been reported. In this respect, the work of Poznansky M. et al (J. Virol 1991, 65, 532-6), Naldini et al (Science, 1996, 272, p 263-7) carried out using the HIV retrovirus and the work of Poeschla E M et al (Nature Medicine, 1998, 4, p 354-7) carried out using the FIV retrovirus can be cited.

The inventors have searched the determinants involved in the mechanism of entry of the retrovirus genome into infected cell nuclei (nuclear import mechanism).

The identification of a triplex DNA determinant essential for import has led the inventors to define novel means, and in particular vectors, for use in transferring genes, or more generally sequences of nucleotides (henceforth termed “transgenes”), into target cells. In particular, the inventors have worked from the HIV (human immunodeficiency virus) retrovirus, a member of the lentivirus family, and have identified and isolated a viral determinant responsible for the nuclear import of proviral DNA of HIV into target cells: central triplex DNA. This DNA triplex has been shown to be able to function in vectors out of the natural context of the HIV-1 genome, as a nuclear import determinant enabling the vector genome to enter the nucleus of target cells.

Mechanisms for retroviral DNA entry into the nucleus exhibit considerable differences from one retroviral family to another. The lentivirus genome is capable of crossing the nuclear membrane of the interphasic nucleus by addressing followed by translocation of its pre-integration complex (linear DNA and associated proteins) through the nuclear pore. Thus such viruses are capable of replicating in the absence of division of the target cell. In particular, they infect differentiated tissue macrophages and dendritic cells, cells at the core of the transmission, dissemination, and the physiopathology of HIV. In contrast, oncovirus genomes and spumavirus genomes are incapable of crossing the barrier constituted by the nuclear membrane. Their pre-integration complex must await mitosis and disorganisation of the nuclear membrane in order to accede to the mitotic chromosomes and be integrated.

The viral determinants responsible for nuclear import of the DNA of the HIV-1 virus have been studied by the inventors. The identification and functional comprehension of the molecular mechanisms of nuclear import of the HIV pre-integration complex is of fundamental importance. The inventors have identified an original mechanism for nuclear import of the HIV-1 genome by which this import is governed by a DNA structure, a triplex at the centre of linear DNA molecules, generated by steps particular to lentiviral reverse transcription.

The triplex DNA structure present at the centre of linear DNA molecules generated during lentiviral reverse transcription, in particular in the HIV retrovirus, has been described by the inventors in different prior publications (Charneau P. et al., J. Mol. Biol. 1994, 241, 651-662; Charneau P et al, Journal of Virology, May 1991, p 2415-2421; Charneau P. et al., Journal of  
5 Virology, 1992, vol. 66, p 2814-2820).

The DNA structure forming a triplex during viral reverse transcription is a polynucleotide comprising a cis-acting central initiation region, or polypurine tract (cPPT), and a cis-acting termination region (CTS), these regions enabling initiation of transcription of a +strand the synthesis of which is initiated by the PPT region present in the centre of the HIV genome or other lentiviruses,  
10 and interruption of synthesis of a second +strand the synthesis of which is initiated at a 3' PPT site upstream of the retroviral LTR (Figure 1).

Formation of the triplex DNA structure is the consequence of a discrete strand displacement event in the retrovirus genome, blocked by the CTS sequence (Charneau P. et al., J. Mol. Biol., 1994).

15 It should be understood that the term "triplex DNA" used here designates a triple-stranded region of DNA, with no reference to the structure of those strands (free displaced strand, or forming a triple helix or a D-loop, etc...).

The structure of the DNA triplex formed during reverse transcription enables or at least contributes to the entry of the retroviral genome into the cell nucleus, thus allowing infection of non  
20 mitotic cells.

Starting from the identification of this required mechanism for entry of the retrovirus into the nucleus of target cells, the inventors have produced a novel generation of lentiviral vector, including the triplex DNA region. The introduction of a DNA fragment from the HIV-1 genome comprising the cPPT and CTS sequences which are cis-acting into an HIV vector system increases transduction  
25 of genes into the cells by stimulating the amount of nuclear import of the vector DNA. This

generation of lentiviral triplex vectors considerably improves transduction of the gene into the cells whether or not they are mitotic.

The invention concerns a nucleotide sequence of retroviral or retroviral-like origin, which can be prepared synthetically, comprising cPPT and CTS regions which are cis-acting in reverse transcription in general, and in particular two associated polynucleotides when they are placed in the normal retroviral genome, each polynucleotide containing at least 10 nucleotides.

The nucleotide sequence of the invention (see Figure 11G where the cis-acting sequences of interest are boxed) comprises on one side, a short nucleotide sequence termed "cPPT" in the case of HIV-1 (minimum 10 base pairs) and on the other side, a sequence termed "CTS" of at least 10 base pairs in the case of HIV-1. The two cis-acting sequences and a nucleotide sequence from a retroviral genome located between these two cis sequences correspond to about 120 nucleotides in the case of the natural HIV-1 genome.

The invention also concerns a nucleotide sequence comprising three DNA strands constituted by, on one hand, the CTS region (or an equivalent region in the case where the origin of the genome used is other than HIV-1 but with the same properties as the CTS region published by Charneau et al., J. Mol. Biol., 1994) and, on the other hand, upstream of the CTS, a region containing about 90 to 110 nucleotides, preferably 99 nucleotides in the case of HIV-1.

The invention concerns a polynucleotide comprising a double stranded DNA fragment corresponding to the cPPT (polypurine tract) region associated with a polynucleotide sequence naturally present in the HIV-1 genome (or an equivalent natural or synthetic sequence), and finally a CTS nucleotide region which adopts a conformation which defines the end of the triple stranded region (3' end) after reverse transcription.

This triple-stranded conformation is termed a "triplex sequence".

By way of example, the triplex sequence is that shown in Figure 11F for HIV-1 or Figure 11G. In vivo, when present in a vector for use for penetrating the nuclear membranes of eukaryotic cells, the triplex stimulates import of DNA into the nucleus of the cell to be modified or transduced.

5 The invention concerns the use of this triplex sequence alone or in a vector to introduce nucleotide sequences to which the triplex sequence is bound into the nucleus of the receiving eukaryotic cell.

Thus the invention provides a recombinant vector, characterized in that it comprises a polynucleotide containing a cis-acting central initiation region (cPPT) and a cis-acting termination region (CTS), these regions being of retroviral or retroviral-like origin, said vector further  
10 comprising a defined sequence of nucleotides (transgene or the sequence of interest) and regulatory signals for reverse transcription, expression and packaging of retroviral or retroviral-like origin.

The term "polynucleotide" used here refers to any nucleic acid sequence in the form of a single or double or triple strand, whether DNA, for example cDNA, or RNA.

By way of example, the invention concerns the transfer of transgenes for therapeutic  
15 purposes, in particular within the context of somatic gene therapy protocols, to insert a nucleotide sequence which modulates or repairs a defective activity in the somatic cells of an organism to rectify poor function of an endogenous gene, or to enable expression of a supplementary function, in particular a function which suppresses the expression or the activity of a gene, for therapeutic purposes.

20 The expression "therapeutic" as used here means the search for or production of a preventative or curative effect, or the search for or production of an improvement or stabilisation of the pathological state of a patient.

Within the context of the invention, and by way of example, the nucleotide sequences termed transgenes or nucleotide sequences of interest can be genes or gene portions or sequences derived  
25 from genes, for example cDNA or RNA. They may also be antisense sequences, negative mutant

sequences of a given gene, or sequences involved in the functions of gene transcription, expression or activation, or sequences suitable for activation of prodrugs or cytotoxic substances.

The activity of the transgene sequences of the invention can also be to stimulate or induce an immune, cellular or humoral response, for example when used to transform cells presenting an antigen.

Thus the invention can be applied to the preparation of vectors used for gene therapy in various domains such as that of hereditary diseases comprising altering a gene, these diseases including Duchenne's muscular dystrophy, cystic fibrosis, neurodegenerative diseases or acquired diseases such as malignant diseases naturally leading to a weak response of the immune system.

The invention can also envisage immunotherapy treatments to stimulate the response to pathogenic agents, for example by the production of CTL, for example in the case of diseases such as cancers or diseases such as AIDS, or to reduce the response against self antigens in the case of autoimmune diseases.

The invention also concerns the provision of means for producing immunogenic compositions or prophylactic or therapeutic vaccines, or immunogenic compositions.

Lentiviral vectors containing a DNA triplex of the invention are also used to construct transgenic animals by transduction of genes into cell lines or embryonic cells.

The vector of the invention contains a transgene inserted under the control of viral or non viral sequences regulating transcription or expression.

The transgene can be included in an expression cassette comprising suitable sequences for regulating its expression in the cell.

A first particularly interesting embodiment of the invention is that in which the recombinant vector is characterized in that the sequences of retroviral origin it contains are derived from the genome of a lentivirus.

Within the context of the present application, the term "derivative" encompasses any sequence identical to the sequence contained in the genome of the retrovirus, or any sequence modified by mutation, insertion, deletion or recombination, provided that it preserves the essential function it possesses in the retroviral genome, with regard to its insertion into the vector of the invention.

Such a sequence could be obtained by any known means enabling identification and isolation of sequences of nucleotides from their organism of origin, in particular comprising the steps of cloning and/or amplification, or by synthesis using any known technique.

Alternatively, the vector of the invention is characterized in that the sequences of retroviral-like origin are derived from a retrotransposon. The retrotransposon yeast TY1 can be mentioned in this regard (Heyman T et al).

The recombinant vector thus described can, for example, be a plasmid recombined by a retroviral or retroviral-like construction and a transgene, if necessary contained in an expression cassette.

The recombinant vector can also be a retrotransposon, a phage, such as a  $\lambda$  phage or a filamentary phage which can be introduced into bacteria, or a vector capable of transforming yeasts such as a YAC.

Such a vector can be used for cell transduction, and in particular packaging of cells and/or target cells, by any method which is known per se, including transfection or infection or transduction, for example by an adenovirus or AAV type vector containing the triplex lentiviral vector.

A vector as defined above can be transcomplemented by one or more additional vectors carrying sequences coding for structure polypeptides from the genome of a selected retrovirus, in particular a lentivirus, or structure polypeptides of a retrotransposon.

In this regard, the vector of the invention can be transcomplemented by providing sequences coding for the polypeptides GAG, POL and ENV, or for a portion of these polypeptides sufficient to enable formation of retroviral particles aimed to vectorise the recombinant vector deprived of viral genes and comprising the transgene the expression of which is sought.

5 A vector of the invention can be characterized in that the transgene or sequence of interest is contained in an expression cassette comprising signals regulating transcription and expression.

In general, the vector(s) used for transcomplementation into retroviral or retroviral-like proteins are depleted in packaging signals.

In this regard, the vectors prepared using the techniques of Goldman et al (1997) for use in  
10 transcomplementation of a recombinant vector of the invention can be cited.

The invention also concerns recombinant retroviral vector particles comprising:

- a) a gag polypeptide corresponding to nucleoproteins of a lentivirus or to functional polypeptide derivatives (GAG polypeptides);
- b) a pol polypeptide constituted by the proteins RT, PRO, IN of a lentivirus or a  
15 functional polypeptide derivative (POL polypeptide);
- c) an envelope polypeptide or functional polypeptide derivatives (ENV polypeptides);
- d) a recombinant nucleotide sequence comprising a defined nucleotide sequence (transgene or a sequence of interest) placed under the control of regulatory signals for transcription and expression, a sequence containing regulatory signals for reverse  
20 transcription, expression and packaging of retroviral or retroviral-like origin and a polynucleotide comprising a cis-acting central initiation region (cPPT) and a cis-acting termination region (CTS), said regions being of retroviral or retroviral-like origin and being inserted in a functional orientation with said regulatory signals of retroviral or retroviral-like origin.

25 The invention also concerns recombinant retroviral vector particles comprising:



- a) a nucleotide sequence termed a gag sequence coding for nucleoproteins of a lentivirus or for functional polypeptide derivatives (GAG polypeptides);
- b) a nucleotide sequence termed a pol sequence coding for the proteins RT, PRO, IN and RN of a lentivirus or for a functional polypeptide derivative (POL polypeptide);
- 5 c) regulatory signals for transcription and expression of the gag and pol sequences;
- d) a nucleotide sequence termed an env sequence coding for envelope polypeptides or for functional polypeptide derivatives (ENV polypeptides), the env sequence being placed under the control of regulatory signals for transcription and expression;
- 10 e) a recombinant nucleotide sequence comprising a defined sequence of nucleotides (transgene), placed under the control of regulatory signals for transcription and expression, a sequence containing regulatory signals for reverse transcription, expression and packaging of retroviral or retroviral-like origin, and a polynucleotide comprising a cis-acting central initiation region (cPPT) and a cis-acting termination region (CTS), said regions being of retroviral or retroviral-like origin, said regions
- 15 being inserted in a functional orientation with regulatory signals of retroviral or retroviral-like origin.

In one variation, the invention provides a nucleotide sequence comprising a polynucleotide comprising a cis-acting central initiation region (cPPT) and a cis-acting termination region (CTS), of retroviral or retroviral-like origin, each of said two regions flanking a concatenation of internal

20 nucleotides, said cis-acting cPPT and CTS regions being inserted into said nucleotide sequence, in a functional orientation with regulatory signals for reverse transcription of retroviral or retroviral-like origin.

GAG and POL polypeptides are nucleoprotein polypeptides from precursors cleaved by viral protease. POL polypeptides comprise reverse transcriptase (RT), protease (PRO), integrase (IN) and

25 Rnase H (RN) of the retrovirus. If necessary, other retroviral proteins are also used to construct

vector particles. It should be noted that the terms "proteins" or "polypeptides" used here encompass the non glycosylated forms or the glycosylated forms of the polypeptides in question.

The gag, pol and env sequences used to construct retroviral vector particles can, if necessary, be modified by mutation, for example by point mutation or by deletion or insertion of one or more nucleotides, or may originate from recombinant chimeras originating from different retroviruses, for example HIV-1 and HIV-2 or HIV-1 and CAEV (Caprine Arthritis Encephalitis Virus), provided that they allow the production of functional polypeptides for the production of viral particles capable of vectorising the transgene to be expressed. In particular, mutated sequences are used to increase the safety of the retrovirus produced.

Advantageously, the recombinant vector of the invention or recombinant vector particles are such that the transgene is under the control of regulatory signals for transcription and expression of non retroviral origin. An example of a promoter which can be used to control expression of the transgene is the CMV promoter, the PGK promoter or EF1 $\alpha$  promoter described by Tripathy, S K et al (PNAS 1994, 91, p 11557-11561).

In a variation of the invention, the transgene can be placed under the control of regulating signals previously identified as being retroviral or retroviral-like in origin, in particular under the control of the LTR sequence.

A lentivirus used to derive the retroviral construction of the invention can be selected from the HIV retrovirus, for example HIV-1, HIV-2 or any different isolate of these two types, or for example from the CAEV (Caprine Arthritis Encephalitis Virus) virus, ELAV (Equine Infectious Anaemia Virus), VISNA, SIV (Simian Immunodeficiency Virus) or FIV (Feline Immunodeficiency Virus).

A particularly advantageous vector of the invention is a vector characterized in that the polynucleotide is a DNA sequence comprising the cis-acting central initiation region (cPPT) and the termination region (CTS) of the genome of an HV1 retrovirus or any other lentivirus.

The central PPT sequence or cPPT sequence is a relatively conserved sequence in lentiviruses and is identified by the presence of numerous purine residues certain of which are shown in Figure 11H. Mutations, even point mutations in one of these regions, can destroy the functional nature linked to the formation of DNA triplex structures.

5        The identification of cPPT sequences is facilitated by the fact that a polypurine sequence located at the upstream edge (5') of the 3' LTR in all retroviruses is repeated in the centre of the genome in lentiviruses. This cPPT sequence can be an exact repeat as in the HIV-1 virus, or slightly modified in other lentivirus (Figure 11H). The central termination sequence CTS has been characterized for the HIV-1 virus (Charneau et al, 1994). It is located about one hundred  
10        nucleotides downstream of the cPPT sequence. In other lentiviruses, CTS sequence candidates are also about a hundred nucleotides (80 to 120 nucleotides) downstream of the cPPT sequence. The probable position of the CTS sequence is indicated for several lentiviruses in Figures 11A to 11E.

      The CTS sequence of the ELAV lentivirus has recently been characterized (Scott R. Stetor et al Biochemistry 1999, 38, p 3656-67). According to the authors, in ELAV, the cPPT and CTS  
15        sequences are respectively

5'AAC AAA GGG AGG GA 3' and

5' AAA AAA TTT TGT TTT TAC AAA ATC 3'.

      Examples of preferred polynucleotides for use in the invention which can be cited are the sequences shown in Figure 11, more precisely the sequences between the two regions cPPT and  
20        CTS, including the sequences in those regions.

      If necessary, the sequence of nucleotides comprising cPPT, the internal polynucleotide (i.e., binding the cPPT to the CTS sequence) and the CTS sequence can be point mutated or mutated by deleting or inserting nucleotides. By way of example, point mutations have been produced in the cPPT sequence of HIV-1 and have shown that it retained residual infectivity in the cells (Charneau  
25        et al, J. Virol. 1992, 66, p 2814-2820).

The invention encompasses any mutated sequence for cPPT or CTS which is at least 60% identical to the natural homologous cis-acting nucleotide sequence from which it originates. In the case of chimeral cis-acting sequences, the percentage is applied to each mutated nucleotide sequence of the chimera.

5        Modifications to the nucleotide sequence of the PPT or cPPT or CTS regions can be introduced to construct the triplex DNA of the invention. Such modifications can reach up to 40% of the natural sequence.

10        The identity of the nucleotide sequences which vary with respect to the natural sequences is calculated strictly with respect to the cPPT or CTS individually and not with respect to the complete triplex DNA nucleotide sequence.

The region between the cPPT and CTS is constituted by a polynucleotide which can either be that found in the original retroviral genome between the CTS and PPT or it can be different therefrom provided that the triplex DNA retains its properties as regards nuclear import of the polynucleotide to enable the nucleotide sequence of interest to be taken inside the nucleus.

15        The polynucleotide of the invention can be introduced into a replicative or non replicative vector. In the case of a retroviral vector, it is a non replicative vector.

In order to prepare large quantities of retroviral vector particles, it is possible to use adenoviral type vectors into which the polynucleotide corresponding to the retroviral genome which contains the triplex DNA sequences and those of the gag, pol and env genes has been introduced.

20        These adenoviral vectors can optionally be rendered replicative by introducing an origin of replication sequence.

Figure 11G shows the cPPT and CTS sequences of HIV-1 in boxes.

In all cases, mutated sequences will be used which retain the capacity to form a DNA triplex during reverse transcription of the genome in the target cell.

A recombinant vector in accordance with a particular implementation of the invention can thus comprise all or a portion of the retroviral or retrotransposon LTR sequences, retroviral PBS sites, and 3'-terminal PPT, the retroviral sequence necessary for packaging of the vector genome in the vector particle. The LTR sequence can be partially deleted, in particular in the U3 region.

5 A particular vector of the invention is the plasmid pTRIP.EGFP which has been deposited at the CNCM (Collection National de Cultures de Microorganismes [National Micro-organism Culture Collection] by the Institut Pasteur, France) on 15<sup>th</sup> April 1998, accession number I-2005. The restriction map for this vector is shown in Figure 10.

10 Another vector in accordance with the invention is the plasmid pTRIP.MEL-IRES-GFP deposited at the CNCM on 20th April 1999, accession number I-2185. This vector is the plasmid pTRIP.MEL-IRES-GFP shown in Figure 14.

A particular recombinant vector of the invention is characterized in that the gag, pol and env sequences are also derived from lentivirus sequences, in particular an HIV retrovirus, more particularly HIV-1 or HIV-2.

15 In a further implementation of the invention, the gag and pol sequences are derived from an HIV retrovirus and the env sequence is derived from a retrovirus which is distinct from HIV or from a virus, for example the vesicular stomatitis virus (VSV).

In general, and as a function of the expression of the transgene which is being researched, an env sequence coding for env polypeptides which are amphotropic with respect to the host in which  
20 the transgene is to be expressed can be selected, or env sequences coding for ecotropic env polypeptides can be selected. The tropism of the env sequence can be a specifically human tropism.

The invention also provides recombinant vector particles comprising a recombinant sequence of nucleotides comprising a defined nucleotide sequence (transgene or sequence of interest) placed under the control of regulatory signals for transcription and expression, regulatory  
25 signals for reverse transcription and expression, a sequence containing regulatory signals for

expression and packaging and a polynucleotide comprising a cis-acting central initiation region (cPPT) and a cis-acting terminal region (CTS).

The invention also provides recombinant vector particles comprising a sequence of recombinant nucleotides comprising a defined nucleotide sequence (transgene) placed under the control of regulatory signals for transcription and expression, regulatory signals for reverse transcription, expression and packaging of a retrotransposon and a polynucleotide comprising a cis-acting central initiation region (cPPT) and a cis-acting terminal region (CTS), these regions being derived from a retrotransposon and inserted in a functional orientation with retrotransposon regulatory signals.

Further, the invention also concerns recombinant vector particles comprising:

- a) a GAG polypeptide corresponding to the nucleoproteins of a retrotransposon or to functional polypeptide derivatives;
- b) a POL polypeptide corresponding to the RT, PRO, IN proteins of a retrotransposon or to a functional polypeptide derivative;
- c) regulatory signals for transcription and expression of gag and pol sequences;
- d) a recombinant nucleotide sequence comprising a defined nucleotide sequence (transgene) placed under the control of regulatory signals for reverse transcription, expression and packaging of a retrotransposon and a polynucleotide comprising a cis-acting central initiation region (cPPT) and a cis-acting terminal region (CTS), said regions being derived from a retrotransposon and inserted in a functional orientation with retrotransposon signal regulators.

Further, the invention concerns recombinant vector particles resulting from expression of:

- a) a nucleotide sequence termed a gag sequence coding for nucleoproteins of a retrotransposon or for functional polypeptide derivatives (GAG polypeptides);

- b) a nucleotide sequence termed a pol sequence coding for the RT, PRO and IN proteins of a retrotransposon or for a functional polypeptide derivative (POL polypeptide);
- c) regulatory signals for transcription and expression of gag and pol sequences, said particles comprising a recombinant sequence of nucleotides comprising a defined sequence of nucleotides (transgene) placed under the control of regulatory signals for transcription and expression, a sequence containing regulatory signals for reverse transcription, expression and packaging of a retrotransposon and a polynucleotide comprising a cis-acting central initiation region (cPPT) and a cis-acting termination region (CTS), these regions being derived from a retrotransposon and inserted in a functional orientation with retrotransposon signal regulators.

The invention further concerns recombinant retroviral-like particles comprising:

- a) a polynucleotide comprising a cis-acting central initiation region (cPPT) and a cis-acting termination region (CTS), said regions being derived from a retrotransposon and inserted in a functional orientation with retrotransposon signal regulators;
- b) a polypeptide corresponding to nucleoproteins of a retrotransposon or to functional polypeptide derivatives (GAGpolypeptides);
- c) a pol polypeptide corresponding to the RT, PRO, IN proteins of a retrotransposon or to a functional polypeptide derivative (POL polypeptide);
- d) a viral envelope polypeptide;
- e) a recombinant nucleotide sequence comprising a defined sequence of nucleotides (transgene or sequence of interest) placed under the control of regulatory signals for transcription and expression, regulatory signals for reverse transcription, expression and packaging of a retrotransposon.

As an example, the invention concerns a recombinant vector as defined above, in which the

regulatory signals for reverse transcription, expression and packaging and the polynucleotide

comprising the cPPT and CTS regions are derived from a retrotransposon, for example a yeast retrotransposon.

In general, the signals regulating transcription and expression of the transgene or sequences coding for structure polypeptides of the vector particle, when they are not retroviral or retroviral-like in origin, are advantageously inducible or conditional signals which are capable of leading to tissue-specific expression.

Recombinant cells characterized in that they are recombined with a vector according to any one of the above definitions are also encompassed by the invention. Recombination can be carried out using any suitable means, in particular transfection or infection, especially transfection or transduction by a vector.

The cells can thus be transiently or stably transfected. They may be packaging cells or target cells, in particular cells in which a therapeutic effect is sought by expression of the transgene.

Particularly interestingly, recombinant cells which are capable of expressing the transgene due to transduction using a vector of the invention are non mitotic differentiated eukaryotic cells.

The invention also encompasses the preparation of recombinant non mitotic primary eukaryotic cells, or mitotic cells.

Examples which can be cited are the cells of the lung, brain, epithelial cells, astrocytes, microglia, oligodendrocytes and neurons, muscle cells, hepatic cells, dendritic cells, neuron cells, bone marrow cells, macrophages, fibroblasts, lymphocytes and haematopoietic cells.

Thus the invention relates to compositions with a therapeutic purpose, characterized in that they comprise a vector as described above, or a recombinant cell defined as indicated above.

The invention also concerns an immunogenic composition comprising a vector as described above or recombinant cells as defined above, said composition being capable of leading to an immune, cellular or humoral response in a given host.



The invention thus provides a polynucleotide as defined above comprising retroviral or retroviral-like cPPT and CTS regions which provides access to its use for nuclear import of a nucleotide sequence (transgene), in particular *ex vivo* in defined cells,

Further, the invention provides a polynucleotide as defined above associated with a  
5 nucleotide sequence of interest or with a transgene.

Finally, the invention concerns the use of a polynucleotide comprising a cis-acting central initiation region and a cis-acting termination region (CTS), these regions being retroviral or retroviral-like in origin, for transfection or transduction of eukaryotic cells with a transgene or polynucleotide of interest.

10 It also concerns the use of a recombinant vector or a polynucleotide of the invention for *in vivo* transduction.

Further characteristics and advantages of the invention will become apparent from the following examples and figures.

## LEGEND TO FIGURES

### 15 **FIG 1: Reverse transcription of lentivirus**

Reverse transcription of lentiviral genomes differs from that of oncogenic retroviruses by the synthesis of the +strand in two distinct halves. A downstream segment is initiated at a central copy of the polypurine tract (cPPT) characteristic of lentiviral genomes. Synthesis of the upstream +strand is terminated after displacement of the discrete strand at the centre of the genome. Blocking  
20 of displacement of the strand by reverse transcriptase is governed by a cis-acting sequence of the HIV genome: the CTS (central termination sequence). The final product of reverse transcription of the lentivirus is a linear DNA carrying a central triple-stranded DNA structure (central triplex) over a length of about one hundred nucleotides.

### **FIG 2: Plasmids used for producing HIV vector particles**

Vector particles were produced by co-transfection of three plasmids: the vector plasmid comprising (pTRIP) or not comprising (pHR) the cis-acting sequences responsible for triplex formation, a packaging plasmid providing, trans, the structural proteins and enzymes of the particle (pCMV $\Delta$ R8.2 or pCMV $\Delta$ R8.91) Naldini et al, 1996 and Zufferey et al, 1997), and a VSV virus envelope expression plasmid (VSV-G).

Only the pertinent parts of the plasmids co-transfected into HeLa cells are shown (Naldini et al PNAS, Oct 1996, Zufferey et al, Nature Biotech, 1997).

The packaging plasmids pCMV $\Delta$ R8.2 or pCMV $\Delta$ R8.91 enable expression of the proteins from gag and pol.

PMD.G codes for the heterologous VSV envelope. The vector plasmids pHR-TRIP were derived from the pHR'CMVlacZ plasmid (Naldini et al): a wild type or mutant triplex sequence has been inserted and the lacZ reporter gene, changed or otherwise in EGFP.

### **FIG 3: Impact of triplex on transduction of EGFP into HeLa cells**

HeLa cells, cultivated in an 8 chamber Labtek, were transduced by different vectors expressing the autofluorescent protein EGFP. The infections were normalised for the quantity of capsid protein (P24 ELISA kit, Dupont) to 2 ng of P24 per inoculum. 48 hours post-infection, the cells were fixed with 1% PBS PFA, mounted in mowiol, then observed with a fluorescence microscope. Three independent fields are shown for the vector of origin with no triplex (HR.EGFP, top), for the vector with triplex (TRIP.EGFP, middle) or for a vector containing a mutated non functional triplex sequence (TRIP D.EGFP, bottom). The right hand side shows the different transductions in the presence of nevirapine, an inhibitor for HIV-1 reverse transcriptase.

### **FIG 4: Quantification of the degree of transduction of the EGFP gene by HIV vectors with or without triplex**

HeLa cells transduced by 2 ng P24 of EGFP vectors with or without triplex were trypsinized 48 hours post-infection. The percentages of cells which were positive for EGFP expression were calculated by flow cytometry (FITC channel). In all cases, transduction was inhibited in the presence of nevirapine, an HIV-1 reverse transcriptase inhibitor. In Figure 4C, the presence of the triplex DNA was observed in the vector stimulated by transduction of GFP (or another gene of interest) in cells in mitosis or non mitotic cells. This transduction was multiplied by a factor of 20 with respect to the results obtained with vectors without a triplex sequence (for example, see Naldini et al, Science, 1996).

**FIG 5: Quantification of the degree of transduction of the LacZ gene by HIV vectors with or without triplex**

The impact of the triplex on transduction was calculated by infecting HeLa cells, cultivated in 96 well trays, using different vectors expressing the lacZ reporter gene. 48 hours post infection, the culture trays were lysed and the beta-galactosidase activity was measured using a luminescent reaction kit (Boehringer). Each transduction was carried out in triplicate with an innoculum normalised to 2 ng of P24.

Upper panel: proliferating HeLa cells.

Lower panel: HeLa cells blocked in their cycle by aphidicoline.

Transduction of the lacZ gene was multiplied by a factor of 6 with a vector containing a triplex sequence with respect to a vector with no triplex sequence.

**FIG 6a & b: Impact of triplex on ex vivo transduction of the EGFP gene in rat spinal primary cells**

Primary explant cells from rat spinal cord were infected with 300 ng of P24 for each vector with and without triplex, and observed using a fluorescence microscope as described above.

**FIG 7: Impact of triplex on in vivo transduction of EGFP gene and luciferase gene in rat brain**

FIG 7-a-1: Transduction at injection site.

The EGFP gene was transferred by direct injection into the striatum of the rat brain of 2 microlitres of the vector corresponding to 50 ng of P24.

Observation of the sections under fluorescence microscopy showed a large transduction of EGFP in the presence of triplex (left hand panel) and very little without (right hand panel).

FIG 7-a-2: Another section representing the experiment described above.

FIG 7-b: **Quantification of impact of triplex on in vivo transduction in the brain.**

FIG 7-b-1: Impact of triplex on transduction of the gene coding for luciferase in in vitro HeLa cells. The graph shows the luciferase production quantified by measuring luminescence (Promega® kit). The presence of the triplex in the vector increased transduction of the luciferase gene by a factor of 8.

FIG 7-b-2: In vivo quantification of luciferase activity in rat brains after injection of vectors coding for luciferase, with or without triplex. The presence of triplex stimulates luciferase transduction by a factor of 8.

FIG 7-b-3: Same experiment as 7-b-2 but carried out in the mouse.

**FIG 8: Strategy for analysis of amount of nuclear import of vector DNA.**

A quantitative test enabling the reverse transcription, nuclear import and integration or circularisation kinetics of the vector DNA in transduced cells was developed. This test advantageously replaced detection by PCR amplification of circles with two LTRs, markers for nuclear import of viral DNA into the nucleus of the infected cell (Bukrinsky et al, Nature 1993, 365, p 666-669). The non integrated linear vector DNA, circular DNAs with one or two LTRs and integrated vector DNA were detected by Southern blot and quantified using a Phosphorimager using the following restriction digestion strategy: the total DNA of the transduced cells was digested with EcoNI and AvaII (two unique sites in the vector genome) then hybridised with a DNA probe generated by PCR precisely spanning the EcoNI site. This probe reacted with different fragments: the internal 0.77 kb fragment, common to all of the vector DNA forms, and for which quantification

using the phosphorimager indicated the total quantity of reverse transcribed vector DNA; a distal fragment of 1.16 Kb specifically indicating the quantity of linear non integrated DNA. After supplemental digestion by the XhoI enzyme, spots with one or two LTRs appeared at 1.4 Kb and 2 Kb respectively. The quantity of integrated vector DNA was calculated by subtracting the signals corresponding to non integrated vector DNA, linear DNA and spots from the signal corresponding to the total reverse transcribed DNA. In the case of a lack of nuclear import, the expected vector DNA profile in the transduced cells is an accumulation of non integrated linear DNA. In contrast, if the vector DNA reaches the nuclear compartment of the cell, the essential part of the linear DNA is integrated into the cellular chromatin and circularises.

#### 10 **FIG 9a: Analysis of nuclear import of vector DNA**

Southern blot analysis 48 hours post transduction in HeLa cells showed a typical lack of nuclear import in the case of the vector without triplex (HR GFP) or containing the triplex sequence in the reverse orientation, which is non functional (TRIP, GFP inv). In the case of these vectors, the signal corresponding to non integrated linear DNA was equivalent to the total DNA signal, indicating that the essential part of the vector DNA remained in the linear form instead of becoming integrated. In the case of the TRIP.GFP vector, the intensity of the signal corresponding to linear DNA was very much lower than the total DNA signal, indicating that a large fraction of vector DNA had been imported into the nucleus and had been integrated therein.

15 **FIG 9-b: Kinetic analysis of degree of nuclear import of vector DNAs with triplex (TRIP-GFP) or**  
20 **without triplex (HR-GFP, TRIPinv-GFP).**

**FIG 9-c: Quantification of state of vector DNA in transduced cells.**

Phosphorimager quantification of the Southern blot of Figure 9b showed that 48 hours post-transduction, the majority of the DNA of the vectors without triplex were in the form of linear non integrated DNA; only a little vector DNA had integrated or circularised. The triplex-free vectors (HR-GFP and TRIPinv-GFP) exhibited a typical lack of nuclear import. In contrast, in the case of

the TRIP-GFP vector, more than 60% of the DNA had integrated into the genome of the transduced cell and only a little vector DNA subsisted in the form of non integrated linear DNA. Introduction of the triplex sequence into the vector had complemented the lack of nuclear import of the HR-GFP vector to the level of the wild type. In fact, the vector DNA profile obtained in the case of the TRIP-GFP vector was comparable with that of a wild type HIV-1 virus. This result shows that the triplex sequence is the only determinant of nuclear import lacking in the HR-GFP construction. Only the integrated form of the DNA vector was active.

**FIG 10:** Restriction map for pTRIP.EGFP vector.

**FIGS 11A-11F:** Polynucleotide sequence comprising cPPT and CTS regions of the CAEV,

EIAV, VISNA, SIV<sub>AGM</sub>, HIV-2<sub>ROD</sub> and HIV-1<sub>LAI</sub> viruses.

**FIG 11G:** represents the triplex DNA sequence of the HIV-1 virus. The cis-acting regions, cPPT and CTS, are boxed and printed in bold capitals.

**FIG 11H:** represents the alignment of cPPT and 3' PPT sequences in several lentiviruses. The top line corresponds to the 3' PPT sequence present in all retroviruses upstream of 3' LTR. The bottom line corresponds to the internal repetition of the PPT sequence termed the cPPT in lentivirus.

**FIG 12:**

Figure 12 represents the production of CTL in vitro from human dendritic cells transduced by the triplex vector with a melanoma CTL polyepitope constituted by epitopes the sequences of which are described in Figure 15 as the gene of interest.

These dendritic cells were brought into contact with mononuclear cells (PBL<sub>0</sub>). The CTL activity was measured after re-stimulation by the corresponding antigenic peptides.

The effective cells/target cells ratio is shown along the abscissa.

**FIG 13:** Cytotoxic response after immunising mice with the TRIP.MEL-IRES-GFP vector.

**FIG 14:** Restriction map for the pTRIP.MEL-IRES-GFP vector.

The *E. coli* strain containing the pTRIP.MEL-IRES-GFP vector was deposited on 20<sup>th</sup> April 1999 at CNCM, accession number I-2185.

**FIG 15:** Sequences for specific CTL HLA A2.1 melanoma epitopes included in the polyepitopic construction of the pTRIP.MEL-IRES-GFP vector. The sequences of the polyepitope are underlined to distinguish each epitope.

**FIG 16:** Very high efficiency transduction of CD34<sup>+</sup> stem cells by triplex HIV vectors.

Flow cytometry (FACS) analysis of transduction of the GFP gene in haematopoietic CD34<sup>+</sup> stem cells by the TRIP-GFP vector. The percentage of CD34<sup>+</sup> cells transduced by the TRIP-GFP vector was more than 85%. This efficiency was notably more efficient than the degree of transduction obtained previously with an HIV vector with no triplex (HR-GFP) in CD34<sup>+</sup> cells (Miyoshi H et al, Science 1999, 283, p 682-6).

## METHOD AND APPARATUS

### Construction of plasmid vectors:

The pTRIP-LacZ and pTRIP-EGFP plasmids derive from the pHR'CMVlacZ construction (Naldini et al, 1996). The lacZ reporter gene of pHR'CMVlacZ was replaced by the ORF of the autofluorescent protein EGFP. The EGFP gene was amplified by PCR from the pEGFP-N1 plasmid (Clontech) using thermostable Pfu polymerase (Stratagene).

The sequences for the PCR primers used were as follows:

Bam EGFP: 5' cc gga tcc cca ccg gtc gcc acc 3'

Xho EGFP: 5' cc ctc gag cta gag tcg cgg ccg 3'

PCR amplification was carried out for 30 cycles under the following conditions:

- denaturation 95°C, 30 sec;
- hybridisation 50°C, 1 min;
- elongation 75°C, 30 sec.

The BamHI and XhoI restriction sites were added at the 5' and 3' end respectively of the EGFP PCR fragment so as to insert it in an orientated manner into the pHR'CMV vector fragment, itself digested with BamHI and XhoI. Insertion of the EGFP PCR fragment using conventional recombinant DNA techniques (Maniatis et al, 1983) generated the pHR-EGFP plasmid.

5 A 184 bp fragment corresponding to the central region of the HIV-1 genome and comprising the cis-acting cPPT and CTS regions responsible for the formation of the triplex during reverse transcription of the HIV was inserted in the ClaI site of the pHR-EGFP and pHR'CMVlacZ plasmids, upstream of the CMV promoter. The central triplex region was amplified by PCR from complete proviral plasmids of the LAI HIV-1 genome comprising the wild type triplex sequence  
10 (pBRU3; Charneau et al, 1991), mutated in the cis-acting termination sequence CTS (pCTS; Charneau et al, 1994) or mutated in the cis-acting central initiation sequence cPPT (p225; Charneau et al, 1992).

The sequences for the PCR primers were as follows:

Nar/Eco TRIP+: 5' gtc gtc ggc gcc gaa ttc aca aat ggc agt att cat cc 3'

15 Nar TRIP-: 5' gtc gtc ggc gcc cca aag tgg atc tct gct gtc c 3'

The PCR reaction conditions were identical to those described above.

The PCR triplex fragments, digested by NarI were inserted into the ClaI site of the pHR GFP and pHR'CMV lacZ plasmids by competitive T4 DNA ligase/ClaI ligation/digestion to eliminate the self re-circularised vector during the ligation step. The orientation of the insertion was analysed  
20 by XhoI/EcoRI digestion, the EcoRI having been introduced into the 5' Nar TRIP+ PCR primer.

The resulting plasmids were termed pTRIP.EGFP in the correct orientation for the triplex and pTRIPinv.EGFP in the reverse, non functional orientation. The vectors comprising a mutated version of the triplex were termed pTRIP X.EGFP, X corresponding to the code of the starting mutant virus (AG, D, CTS or 225) (Charneau et al, J. Mol. Biol. 1994, Charneau et al, J. Virol  
25 1992). Starting from the different plasmids pTRIP.EGFP or pTRIP X.EGFP, the EGFP gene was



replaced by lacZ by orientated XhoI/BamHI exchange. The resulting plasmids were respectively termed pTRIP.Z, pTRIPinv.Z, pTRIP CTS.Z, pTRIP 225.Z.

### **Constructions of HR luc and TRIP luc vectors**

The BamHI-EGFP-XhoI fragment of the RH GFP and TRIP GFP vectors was replaced by the BamHI-Luc-XhoI fragment of the pGEM-luc plasmid (Promega) coding for luciferase.

### **Production of non infectious vector particles**

HIV vectors were produced using a modification of the protocol described by Naldini et al, 1996. The vector particles were produced by transient co-transfection with calcium phosphate of human 293T cells (ATCC), cultivated in a DMEM (ICN), 10% FCS, penicillin, streptomycin medium. Semi confluent 175 cm<sup>2</sup> boxes were simultaneously transfected by three plasmids:

- 15 µg of plasmid coding for the envelope of the vesicular stomatis virus (VSV), pMD.G (Naldini et al, 1996);
- 30 µg of packaging plasmid, pCMVΔR8.2 (Naldini et al, 1996) or pCMVΔR8.91 (Zufferey et al, 1997);
- and 30 µg of the different plasmid vectors pHR or pTRIP.

The calcium phosphate/DNA co-precipitates were left in contact with the cells for 24 hours, the medium was then collected every 24 hours up to post transfection day 3. The cellular debris of the vector supernatants was eliminated by low speed centrifugation. The vector supernatants were stored at -80°C.

### **Concentration of vector particles**

The use of the very stable VSV-G envelope to pseudotype the vector particles enabled them to be concentrated by centrifugation. The supernatant vectors, collected as described above, were ultracentrifuged in 30 ml conical bottom tubes (Beckman) for 90 min at 17000 rpm at +4°C with a

SW 28 rotor (Beckman). The pellets were then taken up in 190  $\mu$ l of PBS, centrifuged for 5 min at 2000 rpm to remove non resuspendable debris, aliquoted and frozen to  $-80^{\circ}\text{C}$ .

### **Transduction of cells in culture**

HeLa cells (ATCC) were transduced by adding vector supernatants, which may or may not  
5 have been ultracentrifuged, in the presence of 10  $\mu\text{g/ml}$  of DEAE dextran. The HeLa cells were cultivated in DMEM medium (ICN) supplemented by 10% foetal calf serum (FCS). HeLa cells were spread in an amount of 20000 cells/well onto a 96 well tray the day before infection then transduced in a final volume of 200  $\mu\text{l}$ . The vector innoculum was normalised to the concentration of capsid protein (P24), calculated using a commercial ELISA test (DuPont). The gene transfer  
10 efficiency was measured using the experimental data for 24 to 48 hours post infection. The amount of transduction of vectors expressing the lacZ reporter gene was revealed either by staining with Xgal in situ (Charneau et al, 1992), or by using a luminometric reaction using a commercial kit (Boehringer) following the manufacturer's instructions. In the case of vectors expressing the reporter gene EGFP, the amount of transduction was qualitatively evaluated by direct observation of  
15 the living cells using a fluorescence microscope, on the FITC channel. The number of cells expressing the EGFP marker was quantified by flow cytometry (FITC channel). The EGFP protein was assayed by measuring the fluorescence of the cellular extract. The 96 well culture plates were rinsed twice with PBS then lysed with 100  $\mu\text{l}$  of 1% NP40 PBS. The EGFP fluorescence was read using a plate fluorimeter (Victor, Wallac) with a 475 nm excitation filter and a 510 nm emission  
20 filter.

HeLa cells stopped in their cell cycle in G1/S transition were prepared under the same conditions as before with prior treatment 24 hours before with transduction with 4  $\mu\text{M}$  of aphidicoline (Sigma). Under these conditions, tritiated thymidine incorporation was inhibited by more than 95%.

### **Ex vivo transduction of primary cells**

Primary rat spinal cord cells were prepared as follows: cords from 13 to 14 day old rat embryos were dissected under a binocular magnifying glass. The tissues were kept in L15 medium (Gibco) supplemented with 3.6 mg/ml of glucose during all the steps. The nerve cells were dissociated by incubating in trypsin (0.05% v/v) for 15 min at 37°C. Trypsin digestion was inhibited by adding 10% foetal calf serum (FCS) and centrifuging at low speed. The cellular pellet was taken up in L15 medium, 3.6 mg/ml of glucose containing 100 µg/ml of DnaseI (Boehringer) by gentle mechanical agitation. The cells were collected by low speed centrifuging through a 4% (w/v) BSA pad.

Spinal cells were seeded onto 24 well plates containing 12 mm diameter glass coverslips coated with poly-DL-ornithine (6 µg/ml) and laminin (3 µg/ml). The cell cultures were maintained in a neurobasal medium (Gibco) containing B27 supplement, 2% FCS, 0.5 mM of L-glutamine, 25 µM of beta-mercaptoethanol and 25 µM of L-glutamate. After 24 hours, the cultures were treated with 10 µg/ml of 5' fluorodeoxyuridine to prevent colonisation of the culture by non neuronal cells.

### **In vivo transduction of EGFP in the rat brain**

Vectors expressing the marker protein EGFP were used for in vivo experiments.

The brains of 5 week old OFA spague dawley rats were injected with 2 µl of HR.EGFP vector or TRIP.EGFP vector. Firstly, the rats were put to sleep by intraperitoneal injection of Imogene 500 (Rhône Merieux). The injections were made into the striatum of each hemisphere using a stereotaxic guide, with a 5 µl Hamilton needle, at a rate of 2 µl/5 min. The rats were sacrificed one week or more after injection, by perfusion of PBS then 2% paraformaldehyde (PFA). The brains were then removed and cut to retain only the portion containing the injection point, visible by the lesion left by the needle. Post fixing with 2% PFA was carried out overnight, followed by cryoprotection with 20% then 30% sucrose. The brains were then covered with tissue-

tek, frozen in solid CO<sub>2</sub> and stored at –80°C. 14 µm slices were made using a cryostat then observed with a confocal microscope.

### **In vivo and in vitro comparison of HR luc and TRIP luc vectors**

One day before transduction, 20000 HeLa cells per well were spread onto 96-well plates.

- 5 Transduction was carried out using the same quantity of vector particles normalised to the P24 content of the preparations: 1 ng of P24 per well, in triplicate, in the presence of 10 µg/ml of DEAE dextran. Two days after transduction, the luciferase activity was measured using a Promega kit (following the manufacturer's instructions) and a Wallac microplate measuring device (Victor).

- 10 The vectors were injected into the brain striatum of OFA spague dawley rats and C57B6 mice. 2 µl of a HR luc or TRIP luc preparation containing 25 ng of P24 was injected (n = 4). The animals were sacrificed 4 days later, the striatum was removed and the luciferase activity was measured using the same technique as before, simultaneously measuring the total protein quantity (Pierce kit).

### **EXAMPLES**

- 15 1. **Fundamental aspects. Nuclear import of pre-integration complex**

#### **HIV-1: Role of central triplex**

- 20 The mechanism for reverse transcription of the HIV virus differs from that of oncogene retroviruses in that the plus strand (+strand) is synthesised in two distinct halves (Figure 1). A downstream segment is initiated at a central copy of the polypurine tract (cPPT), characteristic of lentivirus genomes. Synthesis of the upstream plus strand is terminated after a discrete displacement of the strand at the centre of the genome. Blocking the displacement of the strand by reverse transcriptase is governed by a new cis-acting sequence of the HIV genome: the CTS (central termination sequence). The final product of reverse transcription of lentiviruses is thus a linear DNA carrying a central structure spanning the strand (central triplex) over about a hundred

nucleotides (Charneau et al, 1994). Specific mutagenesis of cPPT or CTS can halt initiation or central termination of synthesis of the plus strand. In both cases, mutant viruses, where the DNA is deprived of the central triplex, are defective for replication.

Analysis of a replicative defect in initiation and central termination mutants has shown that the replicative cycle of initiation mutants or central reverse transcription termination mutants aborts during a posterior step in synthesis of viral DNA and posterior to routing the reverse transcription complex towards the nuclear envelope. When the structure of the viral DNA present in the infected cells is analysed, it is seen that the phenotypes of the initiation and termination mutants are similar. In both cases, the global reverse transcribed DNA content is not affected by mutations in cPPT or CTS. In contrast, an accumulation of linear non integrated DNA is observed, along with very little integrated provirus or circles with 1 or 2 LTRs formed over the same period. Nucleus/cytoplasm fractionation experiments and nucleus permeabilisation experiments have then shown that these linear DNA molecules are associated with the nucleus, but that their integration and/or circularisation can only occur after dissolving the nuclear envelope, which clearly indicates that the viral DNA of the mutants is kept outside this envelope. Further, nuclease attack experiments on the purified nuclei of cells infected with DnaseI immobilised on gold beads again show an accumulation of mutant linear DNA on the cytoplasmic surface of the nuclear membrane. Finally, precise quantification of the integrative capacity of the linear DNA molecules provided or not provided with a wild type central triplex have recently shown that the central triplex does not influence integration of linear DNA into a heterologous DNA target in vitro.

The replicative defect of viruses mutated for initiation or central termination of reverse transcription thus concerns the nuclear import of their pre-integration complex and more precisely the step for translocation through nuclear pores. Lentiviruses, in particular the HIV virus, have developed an original strategy for reverse transcription wherein the aim is to create the triplex at the centre of non integrated DNA molecules, an indispensable determinant for entry of the viral genome

into the nucleus of an interphase cell. This mechanism distinguishes lentiviruses from all other retroviruses wherein access of DNA to the integration site depends on the disorganisation of the nuclear membrane during mitosis.

## 2. Generation of lentiviral vectors containing cis-acting sequences responsible for triplex formation

### 2-1 Principle and importance of lentiviral vectors

The generation of effective lentiviral vectors assumes knowledge of the determinants responsible for active nuclear import and thus infection of non mitotic cells.

The discovery of the involvement of the triplex in the nuclear import of the HIV-1 genome has important consequences for the construction of effective lentiviral vectors. It assumes conservation in the vector construction of cis-acting sequences responsible for the formation of triplex DNA during lentiviral reverse transcription. The vectorological application of this fundamental research consists of adding the central cPPT-CTS region to lentiviral constructions so as to create the triplex DNA structure during reverse transcription of the vector genome. It should be noted that many attempts at constructing a lentiviral vector have been made, based on the same principle as vectors derived from oncovirus (in general MoMLV), and have proved to be disappointing at least as regards the infectious titre. These vectors are replacement vectors, i.e., the ensemble of the viral genome is deleted then replaced, between the two LTRs and the packaging sequence, by the reporter gene or the gene of therapeutic interest (Miller et al, 1989). According to the inventors, this type of construction is not optimal in the case of lentiviral vectors because of the need for the central cPPT-CTS region for nuclear import of the viral DNA. However, HIV vectors constructed on the same principle as retroviral vectors derived from oncovirus, but pseudotyped by the highly fusigenic envelope of the vesicular stomatitis virus (VSV-G) and concentrated by ultracentrifuging, enable in vivo transduction of rat neurons (Naldini et al, 1997; Blomer et al, 1997) and of the liver and differentiated muscle (Kafri et al, 1997). However, complementation

experiments with human pulmonary epithelium xenografts with these HIV vectors coding for the CFTR (cystic fibrosis) gene have proved to be very disappointing. The essential portion of the DNA vector in this tissue remains in the form of linear non integrated DNA, thus revealing a probable defect in nuclear import (Goldman et al, 1997; see the section “Influence of central triplex on the amount of nuclear import of vector DNA”).

## 2.2 Construction and production of “triplex” HIV vectors

In order to test the importance of the triplex structure in a vector system, the inventors took as a basis the constructions described by Naldini et al.. In this system (Figure 2), HIV vector particles are produced by transient co-transfection of three plasmids: a packaging plasmid expressing the whole of the viral proteins with the exception of the HIV envelope, a plasmid expressing the VSV-G envelope and a plasmid vector, pHR-CMVlacZ, comprising the LTRs of HIV, the bipartite packaging signal of HIV and a lacZ expression cassette. Firstly, the lacZ reporter gene was replaced by a gene coding for a highly fluorescent version of EGFP (E green fluorescent protein), more practical for *in vivo* transduction studies. The central region of the HIV-1 LAI genome comprising the cis-acting cPPT and CTS sequences, responsible for triplex formation, was amplified by PCR then inserted into the ClaI site, in the vector construction pTRIP-EGFP. Insertion of the wild type triplex in the correct orientation generated the pTRIP-EGFP vector; in the reverse orientation (*non functional*), it generated the pTRIP<sub>inv</sub>-EGFP vector.

## 2-3 Rapid and sensitive test for detecting a helper virus in lentiviral vector preparations:

### Absence of infectious helper viruses in “vector supernatants”

The production of vector particles from three independent plasmids with a minimum of homologous sequences could minimise the probability of generating a helper virus which was capable of replication. Further, the packaging construction had been deleted for the HIV envelope and for the ensemble of the genes said to be accessory to replication (Vif, Vpr, Vpu, Nef). The packaged vector genome no longer contained HIV other than the 2 LTRs, the sequences necessary

for packaging of the triplex sequence. However, each vector stock was tested for the presence of infectious helper viruses. MT4 cells were infected in triplicate, on a microplate, overnight, then washed extensively and taken up into culture again for 5 days in order to amplify the inoculum. P4 indicator cells (HeLa CD4 LTR-lacZ) were then infected with MT4 cells and their supernatant for 3 days to detect the infectious particles produced. Finally, in situ Xgal staining was carried out. In this manner, any infectious particle produced was detectable in the form of a blue scintillation. This sensitive protocol could detect an HIV inoculum of 0.25 pg of P24, i.e., about 3200 physical particles. Knowing that in the case of HIV, a single particle in 1000 or even in 10000 is infectious, the protocol can probably detect a single infectious particle.

The vector supernatants were systematically deprived of infectious HIV particles.

#### **2-4 Effect of triplex on transduction efficiency by vectors in vitro**

Firstly, (Figure 3), the effect of inserting the central triplex on HeLa cell transduction was measured. HeLa cells were infected with a transfection supernatant from a wild type central triplex vector (TRIP GFP), a vector without this sequence (HR GFP) or with a mutant triplex sequence (TRIP GFP D). The D mutant was a cPPT mutant which prevented central initiation of the +strand and thus the formation of the central triplex (Figure 3). Infections were carried out with supernatants containing the same quantity of particles normalised to the quantity of capsid protein P24.

GFP transduction in HeLa cells was increased in the presence of a wild type triplex and dropped to the base level in the presence of a non functional triplex.

This increase in titre could be quantified using the lacZ reporter gene (Figure 4). These cells were transduced in triplicate by normalising with respect to the quantity of P24 protein. Transductions were carried out using cells blocked or not blocked in division with aphidicoline, which blocks G1/S cells. The vectors used were HRZ (no triplex), TRIP Z (with triplex), TRIP Z



inv (the triplex sequence was in the reverse direction, non functional, and did not lead to formation of a central triplex).

Figure 4A: The gains in transduction of  $\beta$ gal by vectors containing the triplex was 6 to 10 times. It was lost when the triplex was not formed (TRIP Z inv).

Figure 4B: The effect of triplex on transduction of  $\beta$ gal was independent of cell division: similar results were obtained with cells in division or blocked with aphidicoline.

Further, the same results were obtained with HeLa cells, when the packaging plasmid used during production of the vector particles was or was not deleted in the accessory genes Vif, Vpr, Vpu, Nef.

## 2-5 Effect of triplex on efficiency of transduction by ex vivo vectors

The impact of the triplex on EGFP transduction in primary non mitotic cells was then measured. Primary explants from rat spinal cord enriched in neurons were transduced with ultracentrifuged vector supernatants. The transductions were carried out with less than 10  $\mu$ l of ultracentrifuged vector, containing the same number of particles, normalised to the number of ng of P24 capsid protein.

Figure 6: The vector with a triplex sequence transduced a much larger number of rat primary spinal cord explant cells than the vector without triplex.

## 2-6 Impact of triplex on in vivo transduction in the brain

The effect of triplex on in vivo EGFP transduction was then measured by direct injection into the rat brain. The same volume (2  $\mu$ l) of vector supernatant with or without triplex containing the same quantity of P24 protein was injected into the striatum. While a large number of transduced cells was detected in rats injected with the vector with triplex (Figure 7a), it was only possible to detect a few cells expressing EGFP in the brains of rats injected with the vector without triplex, at the exact point of the injection, visible by the lesion left by the needle.

In Figure 7b, the construction of the HIV vectors (with or without triplex DNA sequence) which express the reporter gene luciferase (HR Luc and TRIP.Luc) enabled the impact on gene transduction in the brain to be precisely quantified. In vitro, an increase by a factor of 8 was observed in HeLa cells (Figure 7-b1). An analogous benefit was obtained after direct injection in vivo into the brain striatum of the rat (Figure 7-b2) or mouse (Figure 7-b3).

## 2-7 Impact of triplex on nuclear import of vector genome

A test which enabled the ensemble of the forms of DNA vector in the transduced cell to be followed over time was developed by the inventors: linear DNA, circles with 1 or 2 LTRs but also integrated provirus. This test is based on detecting viral DNA by Southern blot using a cleaving strategy and a choice of probe enabling the different forms of retroviral DNA to be differentiated (see Figure 8). The total DNA of the infected cells or cells transduced by the vectors was digested by one or more restriction enzymes to detach an internal fragment, common to all forms of retroviral DNA or vector DNA present in the cells (linear non integrated DNA, circularised DNA with one or two LTRs and integrated provirus). In the case of a vector, the enzymes selected were Eco NI and Ava II. When using as a probe a fragment generated by PCR exactly spanning the Eco NI site, several bands corresponding to the different forms of DNA appear. The internal fragment enabled the total vector DNA present in the cells to be calculated after quantification using a Phosphorimager. A 1.16 kb fragment corresponded to the distal fragment of non integrated linear DNA, a further 3.3 kb corresponded to non integrated circles. After quantifying the signals with the Phosphorimager, the degree of nuclear import was indicated by the percentage of viral DNA integrated and in the circular form (nuclear viral DNA) with respect to the linear cytoplasmic DNA. The first preliminary blots showed an intracellular DNA profile characteristic of a lack of nuclear import in the case of vectors deprived of triplex or wherein the central region of the HIV-1 genome had been inserted in reverse. The intensity of the signal corresponding to linear DNA was equivalent to that of the total signal DNA 48 hours post infection. In other words, processing of the

DNA vector was mainly blocked in the non integrated linear stage, and very few molecules were integrated (Figure 9). In contrast, in the case of vectors with a triplex, only a little linear DNA subsisted after 48 hours, indicating that the major portion had been imported into the nucleus of the transduced cell, then integrated.

## 5 2-8 Study of the effect of the position of the DNA triplex on vector construction

All lentiviruses contain cis-acting cPPT and CTS sequences responsible for triplex formation during reverse transcription. In all cases, this triplex was found within a few nucleotides of the centre of the linear DNA genome. This central position of the triplex could be important for the optimum function of this determinant of translocation through the nuclear pore. With the vector constructions produced, the triplex sequence had been inserted just upstream of the transcriptional unit of the reporter gene. Depending on the size of the reporter gene, this triplex was found at a greater or lesser distance from the centre of the linear vector DNA genome. In the case of the reporter gene *EFGP* (0.7 kb), the triplex is very close to the centre of the construction; while in the case of *lacZ* (3.1 kb), it is further away (Figure 2). In both cases, the presence of the triplex induced a large gain in titre in the supernatant vectors. Thus there exists a certain “flexibility” in the position of the triplex on the vector genome. However, vectors coding for EGFP have been clearly shown to be more effective than those coding for *lacZ*. It is thus possible that an ideally positioned triplex can result in an additional gain in titre. In order to test this hypothesis, the inventors undertook to clone, in the place of reporter genes, a bank of fragments of random size (partial *Sau3A* digestion), the size distribution of the cloned fragment being analysed before and after transduction of the target cells. If the central position of the triplex is important to its function, constructing a symmetrical vector with respect to the triplex would be important. It is possible to overcome this obstacle by inserting the transcriptional unit of the vector into the U3 region. After reverse transcription, the transgene will be duplicated either side of the triplex before being integrated, thus providing the triplex with a precisely central position.

## **2-9 In vivo transfer in different differentiated tissues**

The capacity of “triplex” lentiviral vectors to efficiently and stably transduce the affected differentiated tissues in various genetic disorders was studied. The potential of these vectors in the brain, and in different tissues such as muscle, pulmonary epithelium and liver in the rat or mouse was studied. Qualitative responses could be obtained relatively rapidly using the EGFP reporter gene. Quantitative measurements of the impact of the triplex on the degree of transduction of these tissues were possible using the reporter gene luciferase. Further, the capacity of these vectors to transduce totipotent stem cells of human haematopoietic tissue could be evaluated, either from purified CD34+ cells or from total cord blood cells.

## **2-10 High efficiency gene transfer in haematopoietic stem cells by triplex HIV vectors**

Haematopoietic stem cells are very important targets for the treatment of a large number of genetic disorders connected with the blood, with muscular disorders or with neurological disorders, and with infectious diseases. The major difficulty for gene transfer by retroviral vectors derived from oncovirus such as MoMLV into these cells is that they only rarely divide and that inducing mitosis by a cytokine treatment is generally accompanied by a loss of totipotency. Figure 16 shows the results of transduction of the GFP gene in CD34 stem cells by the TRIP-GFP vector showing expression of GFP in more than 85% of the cells. The efficiency of transduction of CD34 stem cells by the vector without triplex, HR-GFP, was very low (Miyishi H et al, Science 1999, 283, p 682-6). Since the CD34 stem cells were transduced immediately after their purification, their clonogenic capacity remained intact.

## **2.11 Use of lentiviral vectors with a triplex sequence for transduction of embryonic cells: application to the construction of transgenic animals or modified cell lines**

Retroviral vectors are potentially important tools for the construction of transgenic animals via egg transduction (Rubenstein et al, 1986, PNAS, 83, p 366-368) or ES cells (Friedrich and Soriano, 1991, Genes Dev. 5, p 1513-1523). Using lentiviral vectors can increase the efficiency of

transduction of these totipotent cells. Our preliminary results for transduction of mouse embryo cells via the TRIP-GFP vector show a high efficiency of transfer of the GFP gene but also complete extinction of transcription of the GFP transgene. Certain viral sequences, in particular the primary binding site (PBS), are suspected of intervening in this extinction of expression. In order to overcome this obstacle, self-deleting vectors for these viral sequences, focussed on the CRE/Lox specific recombination system (Choulika et al, 1996, J. Virol. 70, p 1792-98) were constructed.

## 2.12 Immunogenic composition with prophylactic and/or therapeutic applications

### ***A novel immunisation strategy: triplex lentiviral vectors***

#### **Introduction**

The role of T lymphocytes in the antitumoral and antiviral response has been documented in many murine experimental systems and also in man. Different vaccine strategies aim at inducing a protective cytotoxic response against tumours or infectious agents. Lentiviruses have the capacity of crossing nuclear pores and as a result are much better cell transduction vectors. In vitro and/or in vivo cell transduction can lead to the presentation by such cells of epitopes of tumoral and/or viral antigens which as a result induce a specific cellular immunity. For these reasons, the inventors have studied the immunogenic capacity of recombinant triplex lentiviral vectors using either in vitro transduced dendritic cells or direct in vivo administration to “humanised” mice by expression of HLA-A2.1 (Pascolo S et al, 1997). This “HLA-A2.1 pure” HHD mouse is the best animal model for studying the restricted HLA-A2.1 cytotoxic response and has been proposed for carrying out preclinical immunotherapy studies. The whole of our results clearly shows the immunogenic capacity of triplex lentiviral vectors containing tumoral epitopes and thus triplex lentiviral vectors represent a novel immunotherapeutic strategy.

#### **Initial study: comparison of different vaccine strategies**

Different vaccine strategies were initially compared using HHD mice. By arbitrarily selecting 5 tumoral epitopes, the inventors compared five immunisation strategies applicable for

human clinical practice: (i) synthetic peptides in incomplete Freund's adjuvant; (ii) lipopeptides; (iii) recombinant yeast Ty particles in which, independently, the epitopes were fused at the C-terminal end to the P1 protein; (iv) intramuscular administration of naked DNA coding the glycoprotein of the hepatitis B virus fused to epitopes in its pre-S2 portion; (v) intravenous injection of dendritic cells charged with peptides after expansion and differentiation in vitro from marrow cells. Having observed that the injections of particular structures (recombinant yeast Ty) or naked recombinant DNA coding an S glycoprotein of the hepatitis B virus (refer to International patent application WO-A-95/11307, published 25/04/95) were the most effective strategies for inducing cytolytic responses, by inserting a polyepitopic moiety derived from melanoma (10 distinct epitopes) into this glycoprotein, the inventors have documented the possibility of simultaneously inducing cytolytic responses against 5 epitopic peptides out of 10 in all of the test mice.

The particular Ty or naked DNA antigens proved to be effective strategies for inducing cytotoxic responses. However, the large scale production of Ty particles is difficult. Further, it is feared that introducing multiple hydrophobic epitopes into the pre-S2 segment of the hepatitis B virus glycoprotein will entrain a large reduction in the production of particles by CHO cells (the current method for preparing a hepatitis vaccine). The recombinant lentiviruses (HIV-1) produced in the form of largely deleted pseudotypes but conserving the triplex DNA sequence have the capacity to traverse the nuclear membrane of non dividing cells and represent a novel vaccine strategy which is potentially more effective with respect to the vaccine strategies cited above.

## Method, apparatus and results

### Transgenic mice

HHD mice express a monocatenary construction in which the peptide presentation (a1, a2) domains of the HLA-A2.1 molecule are covalently associated at the N-end to the human  $\beta$ -2 microglobulin. The a3 domain and the intracytoplasmic portion of the HLA-A2.1 molecule were replaced by their equivalent in the H-2D<sup>b</sup> molecule (Pascolo S et al, 1997). These mice enabled the

immunogenicity of epitopic peptides and the different vaccine strategies to be studied and compared.

#### **Construction of TRIP-MEL IRES GFP vector**

Firstly, a bicistronic TRIP-IRES-GFP vector was constructed. The EcoRI site of the TRIP-IRES-GFP vector was filled with T4 DNA polymerase creating the TRIP-deltaE-GFP vector. Then a fragment of about 1.2 kb, BamHI-BstXI-SnaBI-EcoRI-IRES-EGFP-XhoI, was cloned in the place of the BamHI-EGFP-XhoI fragment. The fragment containing the IRES-EGFP (internal ribosome entry site) was generously donated by Dr Yongwhon Choi (Rockefeller University, NY, USA). A fragment containing a Kozac consensus sequence and a melanoma CTL polyepitope was generated by PCR, using the pBS meI poly matrix with pfu polymerase and the following oligonucleotides: 5BgImlu MeI: 5' cc aga tct acg cgt gcc acc atg gct gct ggt 3'; 3RIMeI: 5' CG GAA TTC GAC CTA AAC GCA ACG GAT G 3'. The meI PCR fragment was then digested with BgIII and EcoRI and cloned to the BamHI and EcoRI sites of the TRIP-deltaE-IRES-GFP vector creating the TRIP-MEL-IRES-GFP vector.

#### **In vitro transduction efficiency of dendritic cells (DC) by GFP lentiviral vectors with or without triplex**

Murine DCs were obtained from the marrow of transgenic HHD mice in the presence of IL4 and GM-CSF. Human DCs were obtained from healthy HLA-A2.1 haplotype donors (see below). These cells were transduced by LV vectors with or without triplex using different concentrations (75, 150 and 300 ng P24 of lentiviral vector per  $5 \times 10^5$  cells).

The expression of GFP in the DCs was measured by FACS on days 2, 5 and 10. The values in terms of the average fluorescence intensity corresponding to the cell transduction efficiency have shown that triplex lentiviral vectors have 5 to 7 times the transduction capacity for human DCs compared with lentiviral vectors without triplex.

### **Induction of primary CTL responses using human dendritic cells transduced by the TRIP-MEL-IRES-GFP vector**

Immature human DCs were obtained from healthy HLA-A.2.1 haplotype donors in the presence of GM-CSF and IL13 (IDM, Paris, France). Immunophenotyping of these cells by  
 5 monoclonal antibodies against CD1, CD4, HLA-ABC, HLA-DR, CD80, and CD86 showed their immature nature with a DC purity of more than 91%.

The DCs obtained were transduced by the TRIP-MEL-IRES-GFP vector in a concentration of 100 ng P24/vector per  $1 \times 10^6$  cells. The efficiency of DC transduction by TRIP-MEL-IRES-GFP was studied by measuring the expression of GFP by FACS. Mononuclear cells (MNC) from the  
 10 same donor were stimulated by the previously transduced DCs. After three stimulations, the cytotoxic activity of these cells was tested on T2 cells individually charged with 4 epitopic peptides using a conventional 4 hour CTL test. The epitopic peptides Mage-3, gp100.154, GnTV/NA17/A, and tyrosinase 368-D were selected because of their high immunogenicity in the previous experiments.

15 Specific cytotoxic responses were observed against all of the epitopes tested. The lysis percentage for each epitope is shown in Figure 12.

### **Direct immunisation of HHD mice by the TRIP-MEL-IRES-GFP vector**

HHD mice were immunised by 2.5  $\mu$ P24 of the TRIP-MEL-IRES-GFP vector per mouse subcutaneously (SC), intravenously (IV) and intraperitoneally (IP). On immunisation day 11, spleen  
 20 cells from each mouse were individually stimulated by epitopic melanoma peptides for 6 days wherein 2 days were in the presence of 10% TCGF. The lytic activity of these cells was then tested on RMA-S cells charged with the corresponding peptides or on HeLa-HHD cells transduced by the TRIP-MEL-IRES-GFP vector.

The results obtained for each mouse are represented in terms of the specific lysis of RMA-S  
 25 cells (Table 1) and of transduced HeLa-HHD cells (Table 2). The best results were obtained after



administering the vector by SC and IP routes both in terms of lysis and the number of responses simultaneously induced in a given mouse. The remarkable fact is that the majority of mice immunised by the IP route developed cytolytic responses against all the epitopic peptides (Figure 13).

Specific lysates obtained after immunisation of HHD mice by LV containing a melanoma polypeptide. In vitro individual SC stimulation for each mouse on day 8 in the presence of TCGF and peptide											
	Mouse	gp154	gp209	gp280	gp457	G-nTV	Mage-3	Mart 1.27	Mart1.32	Tyro-1	Tyro368-D
SC	1	25	4	13	8	54	17	17	4	4	10
	2	44	1	5	11	89	10	11	4	3	6
	3	39	0	19	21	81	29	26	6	4	0
IV											
	1	7	7	1	8	25	5	8	3	4	3
	2	10	8	0	13	70	13	16	5	9	14
	3	24	6	5	5	65	15	16	3	11	10
	4	5	3	13	12	14	10	5	0	3	0
IP											
	1	30	10	2	3	57	9	6	4	3	2
	2	63	8	7	17	72	11	19	9	7	7
	3	21	7	8	16	72	14	32	0	7	7

Table 1: Specific cytotoxic response after immunisation with TRIP-MEL-IRES-GFP vector

Target cells: RMA85 charged with corresponding peptides

Effector/target ratio: 30

Cytolytic responses of HHD mice immunised by SC, IV or OP routes with the TRIP-MEL-IRES-GFP vector. Results obtained for HeLa-HHD cells expressing the melanoma polypeptide.

	gp154	gp209	gp280	gp457	G-nTV	Mage-3	Mart 1.27	Mart1.32	Tyro-1	Tyro368-D
SC	2	18	15	24	62	15	20	12	20	18
IV	8	10	15	23	50	14	29	10	10	18
IP	24	18	15	25	62	15	32	14	18	20

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Table 2: Specific cytotoxic response after immunisation with TRIP-MEL-IRES-GFP vector

Target cells: HeLa-HHD transduced by the TRIP-MEL-IRES-GFP vector

Effector/target ratio: 30

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## Conclusion

The results demonstrate the capacity of triplex lentiviral vectors to induce highly effective immune responses. Their immunogenic power has been demonstrated not only in vitro on human dendritic cells but has also been evaluated in the transgenic HLA-A2.1 mouse using different modes of administration. Remarkably, specific CTL responses have been obtained for the ten CTL epitopes contained in the melanoma polyepitope. The lysis percentages against melanoma antigens were also higher than those obtained with the same HHD mice with other vaccine strategies such as lipopeptides, recombinant vaccine or DNA vaccination with HBV pseudo-particles. As a result, vaccine strategies based on triplex lentiviral vectors are applicable to a variety of tumoral or infectious disorders.

**BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICRO-ORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE**

**INTERNATIONAL FORM**

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<b>TO:</b>  <b>MADAME D. BERNEMAN</b> <b>Bureau des Brevets et Inventions</b> <b>INSTITUT PASTEUR</b> <b>25-28, rue du Docteur-Roux</b> <b>75724 Paris CEDEX 15</b>  <b>NAME AND ADDRESS OF THE PARTY</b> <b>TO WHOM THE VIABILITY</b> <b>DECLARATION IS ISSUED</b>
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**VIABILITY DECLARATION**  
 issued pursuant to Rule 10.2 by the  
**INTERNATIONAL DEPOSITARY**  
**INSTITUTION**  
 identified on the next page

<b>I. DEPOSITOR</b>	<b>II. IDENTIFICATION OF MICRO-ORGANISM</b>
<b>Name: INSTITUT PASTEUR</b>  <b>Address:</b> <b>Bureau des Brevets et Inventions</b> <b>25-28, rue du Docteur-Roux</b> <b>75015 PARIS</b>	Accession number allotted by the <b>INTERNATIONAL DEPOSITARY</b> <b>INSTITUTION:</b> <p align="center"><b>I - 2005</b></p> Date of deposit or transfer <sup>1</sup> : <p align="center"><b>15 APRIL 1998</b></p>
<b>III. VIABILITY DECLARATION</b>	
The viability of the micro-organism identified under II above was tested on <b>20 APRIL 1998</b> <sup>2</sup> . On that date, the micro-organism was  <input checked="checked" type="checkbox"/> <sup>3</sup> viable  <input type="checkbox"/> <sup>3</sup> no longer viable	

<sup>1</sup> Indicate the date of the initial deposit or, where a new deposit or a transfer has been made, the most recent date (date of the new deposit or date of transfer)

10 <sup>2</sup> In the cases referred to in Rule 10.2 (a) (ii) and (iii), refer to the most recent viability test.

<sup>3</sup> Cross where applicable.

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IV. CONDITIONS USED FOR VIABILITY TEST <sup>4</sup>	
V. INTERNATIONAL DEPOSITARY INSTITUTION	
Name: <b>CNCM</b> Collection Nationale de Culture de Micro-organismes	Signature(s) of person(s) empowered to represent the International Depositary Institution or of authorised official(s): Yvonne CERISIER Administrative Director, CNCM
Address: <b>INSTITUT PASTEUR</b> 28, rue du Docteur Roux F-75724 PARIS CEDEX 15 FRANCE	Georges WAGENER Scientific Adviser for CNCM, bacteria  [signatures]  Date: Paris, 02 June 1998.

<sup>4</sup> To be completed if this information has been requested and if the test results were negative.

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Form BP/9 (second and final page)

**BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICRO-ORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE**

**INTERNATIONAL FORM**

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<b>I. IDENTIFICATION OF THE MICRO-ORGANISM</b>	
DEPOSITOR's identification reference:  <p align="center"><b>pTRIP.EGFP</b></p>	Accession number allotted by the <b>INTERNATIONAL DEPOSITARY</b> <b>INSTITUTION:</b>  <p align="center"><b>I – 2005</b></p>
<b>II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION</b>	
The micro-organism identified under I above was accompanied by:  <input checked="" type="checkbox"/> a scientific description  <input type="checkbox"/> a proposed taxonomic designation (cross where applicable)	
<b>III. RECEIPT AND ACCEPTANCE</b>	
This International Depositary Institution accepts the micro-organism identified under I above, which was received on <b>15 APRIL 1998</b> (date of initial deposit) <sup>1</sup>	
<b>IV. RECEIPT OF REQUEST FOR CONVERSION</b>	
The micro-organism identified under I above was received by this International Depositary Institution on _____ (date of initial deposit) and a request to convert the initial deposit to a deposit in accordance with the Budapest Treaty was received by it on _____ (date of receipt of request for conversion)	
<b>V. INTERNATIONAL DEPOSITARY INSTITUTION</b>	
<b>Name:</b> <b>CNCM</b> <b>Collection Nationale</b> <b>de Culture de</b> <b>Micro-organismes</b>  <b>Address:</b> <b>INSTITUT PASTEUR</b> <b>28, rue du Docteur Roux</b> <b>F-75724 PARIS</b> <b>CEDEX 15</b>	<b>Signature(s) of person(s) empowered to</b> <b>represent the International Depositary</b> <b>Institution or of authorised official(s):</b> <p align="center"><b>Mme Y CERISIER</b>  <b>Administrative Director, CNCM</b>          [signature]</p> <b>Date:</b> <b>Paris, 02 June 1998.</b>

<sup>1</sup> Where Rule 6.4 (d) applies, this date is the date on which the International Depositary Institution was granted recognition.

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Our ref: CNCM-6823.904

Re: Registration of a bacterium with a view to deposit under the terms of the Budapest Treaty.

Copy: *Monsieur Pierre CHARNEAU*  
Viral Oncology Unit

Dear Madam,

We hereby confirm receipt today of twelve frozen samples of the bacterium identified below for the purposes of initial deposit under Rule 6.1 of the Budapest Treaty.

Your request for deposit was recorded at the CNCM

On 20<sup>th</sup> April 1999

With the following number:

*Identification reference*

*Accession number*

pTRIP.MEL-IRES-GFP

**I-2185**

If deposit is accepted, the order number attributed by CNCM will be identical to the accession number and the date of deposit will be the registration date.

Yours sincerely

[signature]

Mme Y. CERISIER

Administrative Director, CNCM



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